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Effects of cold plasma on wheat grain microbiome and antimicrobial efficacy against challenge pathogens and their resistance

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ABSTRACT

The safety and quality of cereal grain supplies are adversely impacted by microbiological contamination, with novel interventions required to maximise whole grains safety and stability. The microbiological contaminants of wheat grains and the efficacy of Atmospheric Cold Plasma (ACP) for potential to control these risks were investigated. The evaluations were performed using a contained reactor dielectric barrier discharge (DBD) system; samples were treated for 0–20 min using direct and indirect plasma exposure. Amplicon-based metagenomic analysis using bacterial 16S rRNA gene and fungal 18S rRNA gene with internal transcribed spacer (ITS) region was performed to characterize the change in microbial community composition in response to ACP treatment. The antimicrobial efficacy of ACP against a range of bacterial and fungal contaminants of wheat, was assessed to include individual isolates from grains as challenge pathogens. ACP influenced wheat microbiome composition, with a higher microbial diversity as well as abundance found on the untreated control grain samples. Culture and genomic approaches revealed different trends for mycoflora detection and control. A challenge study demonstrated that using direct mode of plasma exposure with 20 min of treatment significantly reduced the concentration of all pathogens. Overall, reduction levels for *B. atrophaeus* vegetative cells were higher than for all fungal species tested, whereas *B. atrophaeus* spores were the most resistant to ACP among all microorganisms tested. Of note, repeating sub-lethal plasma treatment did not induce resistance to ACP in either *B. atrophaeus* or *A. flavus* spores. ACP process control could be tailored to address diverse microbiological risks for grain stability and safety.

1. Introduction

Cereal grains have been a principal component of the human diet for thousands of years and remain the most important contributor to human food supplies globally. More than 50% of world daily caloric intake comes directly from cereal grain consumption (Awika, 2011; Enghiad et al., 2017). Wheat is one of the world's leading food crops and the most grown cereals – it is not only a major source of starch and energy, but constitutes a substantial source of protein, B vitamins, dietary fibre, and phytochemicals that are essential or beneficial for human health (Shewry and Hey, 2015). *World population* is *expected* to be 50% larger than at present by 2050 and global grain demand is projected to double (Tilman et al., 2002). Fulfilling the food demand of an increasing population is therefore a major global concern, especially due to the fact that more than one-third of food is lost or wasted in postharvest operations. Up to 50%–60% of cereal grains can be lost during processing caused mainly by presence of microorganisms and insects (Kumar and Kalita, 2017), rendering the cereals unsuitable for human or animal consumption.

The possible sources of contamination include air, dust, water, soil, insects, birds and rodents, as well as storage and shipping containers, and handling and processing equipment (Bullerman and Bianchini, 2009), therefore the entire chain is exposed. A substantial proportion of cereal grains (25%–40%) worldwide are contaminated by mycotoxins, the toxic secondary metabolites produced by filamentous micro-fungi or moulds (Bullerman and Bianchini, 2009; Kumar and Kalita, 2017). Aflatoxins, produced by two species of fungi, *A. flavus* and *A. parasiticus*, are often considered the most dangerous group of mycotoxins (Sawicka, 2019).

The application of chemical insecticides and fumigants during grain

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storage can lead to the accumulation of pesticides and fumigant residues in treated grains, as well as development of fungicide and insecticide resistance. There is growing interest in developing new technologies for cereal grain preservation and decontamination that are environmentally and societally acceptable (A. Los et al., 2018b; Scholtz, 2019). Atmospheric cold plasma (ACP) treatment has been evaluated in various studies as a successful technology for both removal of surface contamination and stimulation of seed germination (comprehensively reviewed by Scholtz, 2019).

The aim of this work was to understand the potential of ACP treatment for microbiological control of wheat grains. To characterize the composition of the fungal and bacterial communities before and after treatment, amplicon-based high-throughput sequencing (HTS) was performed. The antimicrobial efficacy of ACP was tested against a range of grain contaminants including both bacteria and moulds artificially inoculated on wheat grains. The potential for sporulation enhancement or resistance development after sub-lethal plasma treatment was investigated using *B. atrophaeus* and/or *A. flavus*.

2. Materials and methods

2.1. Wheat samples

Organic wheat grains (*Triticum aestivum* L., origin: United Kingdom) were purchased from a local organic store (Dublin, Ireland) and used to study the influence of ACP treatment on grains microbiome and for isolation of contaminants for challenge study. To investigate the effect of ACP on individual challenge microorganisms, wheat grains were sterilized by autoclaving at 121 °C for 15 min and stored for 24 h in 50 °C to obtain constant moisture content of the grains. Subsequently, the grains were stored at room temperature. The absence of background microbiota after autoclaving was confirmed by spread plating method according to the procedure described previously by Los et al. (2018a).

2.2. Isolation and identification of microorganisms from wheat grains

Dilution plating technique: The isolation of wheat mycoflora was performed by the dilution plating technique described by Pitt and Hocking (2009) with minor modifications. Wheat grains (1 g, $n = 10$) were transferred in a test tube containing 9 ml of sterile deionised water and soaked for 30 min. The grains were crushed using a sterile glass rod and then shaken for 30 min at 180 rpm. Resulting suspension was diluted and aliquots of appropriate dilutions were spread plated on tryptic soy agar (TSA, Biokar Diagnostics, France) and potato dextrose agar (PDA, Biokar Diagnostics, France). The plates were incubated for 48 h and 7 days at 37 and 25 °C, respectively. After incubation, bacterial and fungal isolates were sub-cultured on TSA and PDA, respectively, and stored at 4 °C. For long-term storage, bacterial and fungal spore suspensions were maintained at −80 °C in 50% glycerol. For tentative fungal genera identification, macroscopic and microscopic morphological features of fungal isolates were compared with those described by Pitt and Hocking (2009). The isolates were identified to species level using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). For the analysis, bacterial and fungal biomass was prepared according manufacturer instructions (VITEK®MS Mould Kit, Biomérieux). Briefly, each bacterial and fungal isolate was grown on TSA and PDA at 37 and 25 °C for 24 h and 7 days, respectively, in duplicate. For protein extraction, the fungal cultures were subjected to ethanol, 70% formic acid, and acetonitrile precipitative steps. The bacterial culture or fungal supernatant from the final step was smeared or deposited on a target slide in duplicate ($n = 4$), overlaid with an α-cyano-4-hydroxy cinnamic acid matrix (VITEK® MS-CHCA, Biomérieux, France) and analysed using MALDI-ToF MS (VITEK® MS, Biomerieux, France) with spectra obtained in a mass range of 2,000–14,000 m/z (Da). *Escherichia coli* ATCC 8739 was used as a calibration strain. The obtained mass spectra were compared with the reference mass spectra of the VITEK® MS database (V3.1, BioMérieux, France). The isolates were considered as identified at identification confidence (IDC) level of 66.6–99.9% (unless otherwise stated). Among nine bacterial and twelve fungal strains isolated from wheat grain, six and seven, respectively, were identified as *Bacillus atrophaeus/subtilis*, *Bacillus horneckiae*, *Bacillus pumilus*, *Actinomyces turicensis* (48.7%), *Pantoea agglomerans*, *Staphylococcus hominis* and *Aspergillus flavus*, *Aspergillus candidus*, *Aspergillus versicolor*, *Penicillium chrysogenum, Penicillium bialowiezense* (52.8%), *Curvularia hawaiiensis/Alternaria alternata* (50/50%), *Alternaria alternata*.

2.3. Microbial cultures

In total, one bacterial and six fungal strains were used as challenge microorganisms for inoculation of grains. *Bacillus atrophaeus* var. *niger* ATCC 9372 and *Aspergillus niger* ATCC 16404 were obtained from School of Food Science and Environmental Health, Technological University Dublin (TU Dublin). *Penicillium citrinum* DSM 1179 and *Penicillium verrucosum* DSM 12639 were obtained from Leibniz Institute, German collection of microorganisms and cell cultures (DSMZ) and resuscitated according manufacturer instructions. Three fungal strains isolated from wheat grains, namely *A. flavus/oryzae*, *A. candidus* and *P. chrysogenum* were selected for inoculation studies (Fig. 1). According to morphological features and characteristic yellowish-parrot green colonies colour, the *A. flavus/oryzae* was identified as *A. flavus*, which is consistent with descriptions of Zulkifli and Zakaria (2017) and Iheanacho et al. (2014). In addition, the isolate's colony morphology appeared different to that of commercial *A. oryzae* strain available at TU Dublin culture stock, whose aging colonies changed to brown colour whereas *A. flavus*'s remained green (Chang et al., 2014). Morphological characteristics of *A. candidus* and *P. chrysogenum* were similar to the description of Varga et al. (2007) and Abastabar et al. (2016), respectively. All microorganisms were routinely sub-cultured on corresponding media and maintained at 4 °C.

2.4. Inoculation of grain

Inoculum preparation: *B. atrophaeus* vegetative cells and endospore suspensions were prepared according to the procedure described in our previous work (Los et al., 2018). Briefly, *B. atrophaeus* cells were harvested by centrifugation at 10,000 rpm for 10 min and washed twice in PBS. The bacterial density was determined by measuring absorbance at 550 nm using the McFarland standard (BioMerieux, Marcy-l'Etoile, France) and a working inoculum corresponding to a concentration 8.0 $\log_{10}$ CFU/ml was prepared in PBS. To prepare endospore suspension, *B. atrophaeus* was inoculated on TSA supplemented with 3.0 mg/l of manganese sulphate and incubated at 30 °C for up to 10 days. The spores were collected by flooding the agar with sterile PBS. To inactivate vegetative cells, resulting suspension was heat-shocked for 20 min at 80 °C, washed twice in PBS at 4 °C and finally re-suspended in sterile ice-cold PBS.

For preparation of fungal spore suspensions for *A. niger*, *A. flavus*, *A. candidus*, *P. citrinum*, *P. verrucosum* and *P. chrysogenum*, PDA plates were inoculated with selected strain and incubated at 25 °C until good sporulation was obtained. The spores were harvested by flooding the agar surface with 10 ml of sterile phosphate buffered solution (PBS, Oxoid LTD, UK) containing Tween 20 (1%) and scraping the spores from mycelia using a sterile spreader. The suspensions were washed twice in sterile PBS prior to use.

Working inocula for each test microorganism were prepared in PBS with concentration corresponding to an average of 8–9 decimal logarithmic units of colony forming units per ml ($\log_{10}$ CFU/ml). The concentration of each inoculum was confirmed by spread plating method. For inoculation, sterilized wheat grains were transferred into sterile Petri dishes (10 g) and inoculated with a suspension of selected microorganism (0.5 ml). The grains were mixed by shaking for

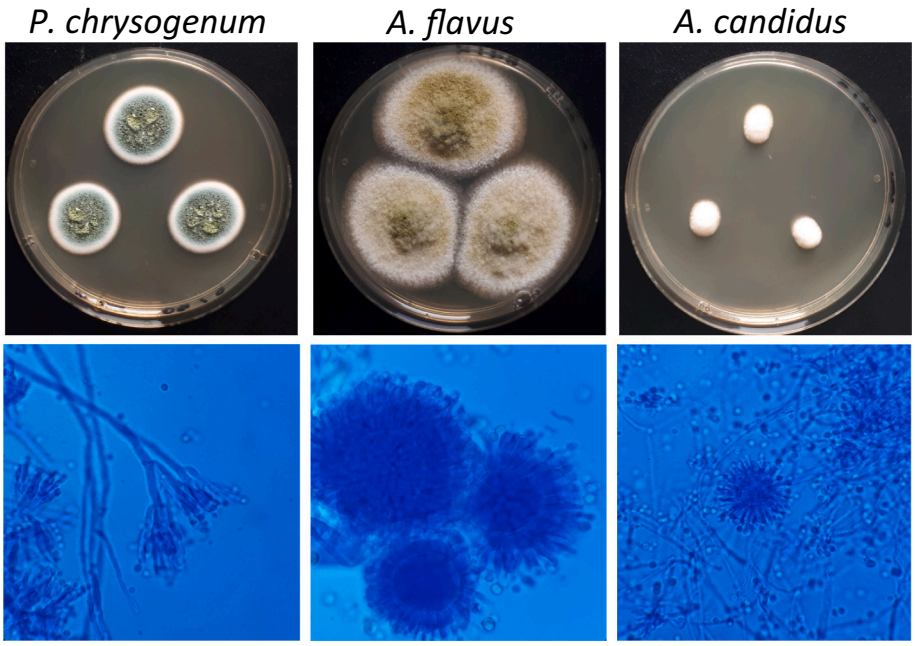


Fig. 1. Wheat fungal isolates on PDA after incubation for 7 days at 25 °C. Top panel – colonial morphology and bottom panel – optical microscopy images (magnification 1000 X).

approximately 30 s to ensure even distribution of cells. Prior to treatment, inoculated grains were left in biological safety cabinet for 1 h to allow attachment to grains surface and to achieve the moisture content of grains as before inoculation (12.8%).

2.5. ACP system set-up

The ACP system used was a high voltage (HV) dielectric barrier discharge (DBD) system with a maximum voltage output in the range 0–120 kV_{RMS} at 50 Hz, described in detail by Pankaj et al. (2013) and fully characterised by Moiseev et al. (2014) and Milosavljević and Cullen (2017). The samples were subjected to contained ACP treatment at 80 kV under atmospheric pressure with air as the working gas. The total distance between the two aluminium disk electrodes (diameter 15 mm) was approximately 50 mm, which was equal to the height (20 mm) of the polypropylene container (310 × 230 mm) utilised as a sample holder and the thickness of the top (10 mm) and the bottom (7 mm) Perspex dielectric barriers. Samples were placed inside the container and subjected to either direct (within the area of plasma discharge) or indirect (outside the area of plasma discharge) mode of plasma exposure as described (Los et al., 2017). Both direct and indirect treatments were achieved simultaneously, i.e. two samples per container: one for direct and one for indirect treatment. Before treatment, each container was sealed with a high barrier polypropylene bag (B2630 Cryovac, Ireland).

2.6. ACP treatment

Un-inoculated grains were used to investigate the effect of treatment on grains microbiome and inoculated wheat grains were used to quantify plasma inactivation rates. Samples (2 g) were exposed to direct/indirect ACP treatment for 5 or 20 min. After treatment, the samples were stored unopened at 15 °C for 24 h of post treatment retention time (PTRT). Untreated control samples were retained under identical storage conditions. Unless otherwise stated, all experiments were performed in duplicate and replicated three times. For grains microbiome sequencing, five plasma treated samples were combined and analysed as a single sample.

2.7. Amplicon-based metagenomic analysis

DNA extraction: Un-inoculated, either ACP treated or untreated control wheat grains (10 g) were soaked in sterile maximum recovery diluent (MRD, Scharlau, France) for 30 min, crushed and stomached in double strength stomacher bags for 30 min. The suspension was then used for isolation of microbial genomic DNA using PowerFood®Microbial DNA Isolation Kit (QIAGEN ®, Ireland) according to manufacturer instructions. DNA concentration was verified using dsDNA BR Assay Kit (Invitrogen™ Qubit™) and Qubit fluorimeter and the presence of the extracted DNA was verified on 0.7% agarose gel.

Amplicon Generation: The 16S rRNA, 18S rRNA genes and internal transcribed spacer (ITS) of distinct regions 16SV4/16SV3/16SV3-V4/16SV4-V5, 18SV4/18SV9, ITS1/ITS2, respectively, were amplified using specific primers: bacterial 16S 341F (5'-CCTAYGGGRBGCASCAG-3′) - 806R (5'-GGACTACNNGGGTATCTAAT-3′), archaeal 16S U519F (5'-CAGYMGCCRCGGKAAHACC-3′) - 806R (5'-GGACTACNSGGGTMTCTAAT-3′), fungal 18S 528F (5'-GGCGGTAATTCCAGCTC CAA-3′) - 706R (5'-AATCCRAGAATTTCACCTCT-3′), fungal ITS ITS5-1737F (5'-GGAAGTAAAAGTCGTAACAAGG-3′) – ITS2-2043R (5'-GCT GCGTTCTTCATCGATGC-3′) with the barcode, to analyse bacterial and fungal taxa, respectively. All PCR reactions were carried out with Phusion® High-Fidelity PCR Master Mix (New England Biolabs). PCR product was then used to verify amplification by 2% agarose gel electrophoresis. Samples with bright main strip between 400 bp–450 bp were chosen for further experiments.

The products of triplicate PCR reaction from one sample were mixed at equal density ratios and purified with Qiagen Gel Extraction Kit (Qiagen, Germany). The libraries were generated with NEBNext® UltraTM DNA Library Prep Kit for Illumina and quantified via Qubit and Q-PCR, for analysis by Illumina platform.

Sequencing data processing: Paired-end reads was assigned to samples based on their unique barcodes and truncated by cutting off the barcode and primer sequences. Paired-end reads were merged using FLASH (V1.2.7, http://ccb.jhu.edu/software/FLASH/), which was designed to merge paired-end reads when at least some of the reads overlap the read generated from the opposite end of the same DNA fragment, and the splicing sequences were noted as raw tags. Quality filtering on the raw tags was performed under specific filtering conditions to obtain the

high-quality clean tags according to the Qiime (V1.7.0, http://qiime.org/scripts/split_libraries_fastq.html) quality controlled process. The raw tags were compared with the reference database (Gold database, http://drive5.com/uchime/uchime_download.html) using UCHIME algorithm (UCHIME Algorithm,http://www.drive5.com/usearch/manual/uchime_algo.html)[17] to detect chimera sequences (https://drive5.com/usearch/manual/chimeras.html). Chimera sequences were removed, and the Effective Tags were obtained.

OTU cluster and Taxonomic annotation: Sequence analyses were performed by Uparse software (Uparse v7.0.1001 http://drive5.com/uparse/) using the effective tags. Sequences with ≥97% similarity were assigned to the same OTUs. Representative sequence for each OTU was screened for further annotation. For each representative sequence, Mothur software was performed against the SSUrRNA database of SILVA Database (http://www.arb-silva.de/) for species annotation at each taxonomic rank (Threshold:0.8– 1) (kingdom, phylum, class, order, family, genus, species). To obtain the phylogenetic relationship of all OTUs representative sequences, the MUSCLE program (Version 3.8.31,http://www.drive5.com/muscle/) was used to facilitate rapid comparison of multiple sequences. OTUs abundance information were normalized using a standard of sequence number corresponding to the sample with the least sequences. Subsequent analysis of alpha diversity and beta diversity were performed on the basis of the normalized data output. According to the analysis result of OTUs clustering, the OTU table was normalized, both the common and unique information for different samples were analysed, and the Venn and Flower diagram was generated.

Statistical analysis: Alpha diversity was applied to analyse biodiversity complexity for a sample through 6 indices; namely Observed-species, Chao1, Shannon, Simpson, ACE and Good-coverage. These indices were calculated with QIIME (Version 1.7.0) and displayed with R software (Version 2.15.3). Beta diversity represents the explicit comparison of microbial communities based on their composition. Beta-diversity metrics thus assess the differences between microbial communities. To compare microbial communities between each pair of community samples, a square matrix of “distance” or “dissimilarity” was calculated to reflect the dissimilarity between treated and untreated control samples, such as Unweighted Unifrac and Weighted Unifrac distance. Beta diversity on both Weighted and Unweighted Unifrac was calculated by QIIME software (Version 1.7.0). Cluster analysis was preceded by principal component analysis (PCA), which was applied to reduce the dimension of the original variables using the FactoMineR package and ggplot2 package in R software (Version 2.15.3).

2.8. Effect of ACP on inoculated grain

Microbiological culture analysis: ACP treated and untreated control wheat grains (1 g) were transferred into separate sterile stomacher bags containing 10 ml of sterile MRD and stomached for 10 min. Samples inoculated with bacterial endospores were heat-shocked for 20 min at 80 °C and cooled in ice for 10 min to inactivate the vegetative cells prior to microanalysis. Samples were serially diluted in MRD and aliquots (0.1 ml or 1 ml) of appropriate dilutions were plated on corresponding media: TSA was used to estimate the recovery of vegetative cells and spores of *B. atrophaeus*, while PDA was used for fungi. TSA and PDA plates were incubated at 37 °C for 24–48 h and at 25 °C for 5–7 days, respectively. Results were expressed as viable counts per gram of grains ($\log_{10}$ CFU/g) and reduction factor ($\log_{10}$). Reduction factor was calculated as the difference between the viable counts for untreated samples and samples subjected to ACP treatment.

Capacity of ACP to induce bacterial sporulation: Plasma treatment time of 5 min was considered as sub-lethal treatment and was used to study the capacity of ACP to induce sporulation in *B. atrophaeus*. After ACP treatment and PTRT of 24 h, wheat grains inoculated with vegetative cells of *B. atrophaeus* were incubated at either 15 °C – to reflect appropriate grain temperature storage conditions or 30 °C – optimal temperature for bacterial sporulation. The number of total viable cells and spores was monitored on day 0, and after 7 and 21 days of incubation. Sporulation was monitored by enumerating cells resistant to an 80 °C heat treatment for 20 min. The number of vegetative cells was calculated by subtracting the number of sporulated cells from the total bacterial count. The percentage of spores in total bacterial count was calculated according to the formula:

$$\textit{Precentage spores} = \frac{\textit{spore count } [\textit{number of spores}]\ x\ 100\%}{\textit{total bacterial count } [\textit{number of vegetative cells and spores}]}$$

2.9. Microbial resistance studies

To study the possible development of resistance to ACP treatment, *B. atrophaeus* vegetative cells and *A. flavus* spores were used. Wheat grains were inoculated with microorganisms according to the procedure described in methodology Section 2.4. Inoculated samples were exposed to direct/indirect 5 min of ACP treatment and PTRT of 24 h and subjected to microbiological analysis by plating appropriate dilutions on TSA (methodology Section 2.8). Survivor bacterial colonies were subcultured in TSB at 37 °C for 24 h at 180 rpm and used for inoculation of grains and subsequent ACP treatment. Again, colonies recovered on TSA were subcultured in TSB for grain inoculation and treatment. This procedure was repeated for five consecutive cycles. Similarly to *B. atrophaeus*, samples inoculated with *A. flavus* spores were subjected to repetitive ACP treatment and PTRT, where for grain inoculation, spore suspension was prepared from colonies formed on PDA as described in methodology sections 2.4 and 2.8. Each biological sample was duplicated and each of five consecutive ACP treatments was repeated three times ($n = 6$). Control samples consisted of inoculated untreated grains subjected only to PTRT of 24 h at 15 °C.

2.10. Statistical analysis

Statistical analysis was performed using IBM SPSS statistics 21 Software (SPSS Inc., Chicago, USA). Means of ACP-treated samples and untreated controls were subjected to analysis of variance (ANOVA) and compared according to the method of Fisher's Least Significant Difference (LSD) at the 0.05 level.

3. Results

3.1. Effect of treatment on microbial community composition and structure

Amplicon-based metagenomic analysis: Amplicon was sequenced on Illumina paired-end platform to generate 250 bp paired-end raw reads, and then merged and pre-treated to obtain clean tags. The chimeric sequences in clean tags were detected and removed to obtain the effective tags, which were used for subsequent analysis. The summarizations obtained in each step of data processing are shown in Supplementary Table 1. In the process of constructing OTUs, basic information of different samples was collected, such as effective tags data, low-frequency tags data and tags annotation data (Supplementary Fig. 1).

3.1.1. Relative abundance

According to the taxonomic annotation results, the top ten taxa of each sample were selected to form the distribution histogram of relative abundance of taxa with higher relative abundance and their proportion in different classification levels for each sample (Fig. 2). At phylum level, bacterial OTUs were dominated by *Proteobacteria*, *Cyanobacteria*, *Firmicutes* and *Bacteriodetes*. *Proteobacteria* was the most abundant phylum in the control wheat sample (76%), and this decreased to 50/

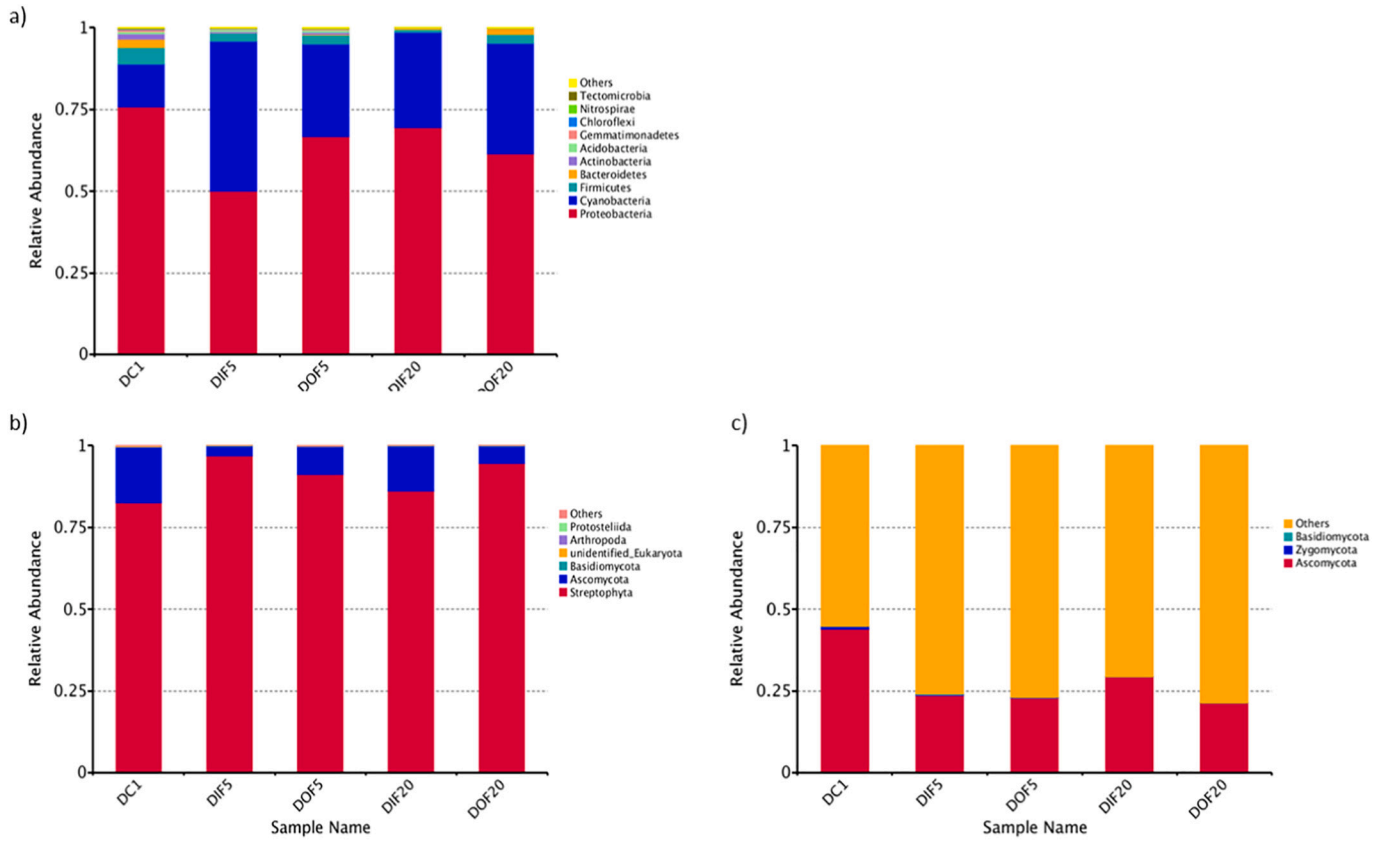


Fig. 2. Taxa relative abundance in phylum for a) bacterial 16S and fungal b) 18S and c) ITS conservative regions. Y-axis represents "Relative Abundance" and X-axis represents "Samples Name". "Others" represents a total relative abundance of the rest phyla besides the top 10 phyla. DC1 - untreated control grains, DIF5/DOF5 and DIF20/DOF20 - directly/indirectly plasma treated grains for 5 and 20 min, respectively.

67% and 69/62%, after direct/indirect mode of plasma exposure for 5 and 20 min, respectively (Fig. 2a). *Proteobacteria* consisted mainly of *Alphaproteobacteria* (74%), *Gammaproteobacteria* (19%) and *Betaproteobacteria* (7%) as detected on untreated control samples. Post plasma treatment, the dominance of *Alphaproteobacteria* increased, while *Gammaproteobacteria* decreased to 1/8% and 1/2% on 5 and 20 min directly/indirectly treated samples, respectively. *Betaproteobacteria* decreased to ~0.2–2% post treatment. The abundance of both *Firmicutes* (5%) and *Bacteriodetes* (3%) phyla on the control grains decreased to 0.2–2% or was not detected after treatment, while the dominance of *Cyanobacteria* increased, proportionally (Fig. 2a). In all samples approximately 82–96% of the 18S rRNA sequences were assigned to *Streptophyta triticum* and the remaining sequences to kingdom fungi. The relative abundance of fungi decreased from 17% on the control to 3/9% and 14/5% after 5 and 20 min of direct/indirect treatment, respectively (Fig. 2b). A higher proportion of the fungi domain was associated with ITS approach, with 45% detected on the control sample and 21–29% detected on plasma treated grains and the remaining taxa were assigned to unclassified eukaryotes (Fig. 2c). While both 18S rRNA and ITS sequencing identified major phyla *Ascomycota* and *Basidiomycota*, *Zygomycota* was detected only with ITS reads. Again, untreated grains had a higher relative abundance for *Zygomycota* (2%) compared with plasma treated grains (0.02–0.9% of fungi). Among fungi, *Ascomycota* was the most dominant phylum (~ 98–100% of fungi found on all samples with both 18S and ITS), which relative abundance decreased from 44% (of root) on the control grains to 21–29% on the treated samples (Fig. 2c). The phylum *Basidiomycota* was present in a lower, but similar abundance levels in the community (~ 0.01–0.3%) across all treated and untreated samples detected with both 18S rRNA and ITS targets. With 18S rRNA, *Ascomycota* were dominated by the class *Dothideomycetes* (79–88%) with most abundant genus *Cochliobolus* (79–84%) following by *Cladosporium* (8–11%) and *Boeremia* (7–11%), phylum *Sordariomycetes* (8–17%) with classified genus *Fusarium* and phylum *Eurotiomycetes* (2–9%) with genus *Aspergillus* accounting for 100%. Similar to 18S, ITS sequencing illustrated the dominance of phylum *Dothideomycetes* (82–94%) where at the genus level *Alternaria* accounted for 97%. Similarly to 18S, *Dothideomycetes* phylum was followed by *Sordariomycetes* (4–16%) and *Eurotiomycetes* (2–6%), however, if compared to 18S, different genera were identified.

3.1.2. Taxonomic abundance cluster heatmap

According to abundance information of top 35 genera of all samples, the heatmap was generated to examine the similarity and difference between the samples and whether the samples with similar processing are clustered or not (Fig. 3). As can be seen, bacterial genera belonging to *Bacteroidetes*, *Firmicutes* and *Proteobacteria* were richer on the untreated control (Fig. 3a). Similarly, for OTUs of 18S and ITS regions, a higher number of more abundant genera were observed on the control than on treated samples (Fig. 3b and c).

3.1.3. Alpha diversity indices

Alpha diversity was applied to analyse complexity of the microbial community within each sample. OTUs generated at 97% sequence identity were considered to be homologous in species. Statistical indices of alpha diversity for each individual sample are summarized in Supplementary Table 2 (number of reads chosen for normalization: cut off a) 29,955 and b, c) 119,015). Fig. 4 represents boxplots of observed species and Shannon indices for the control samples and for 5 min- and 20 min-treated groups (direct and indirect modes are combined for each treatment time point). The number of observed bacterial and fungal species and corresponding Shannon indices was always lower for control samples. As observed with 16S and ITS genes, the number of

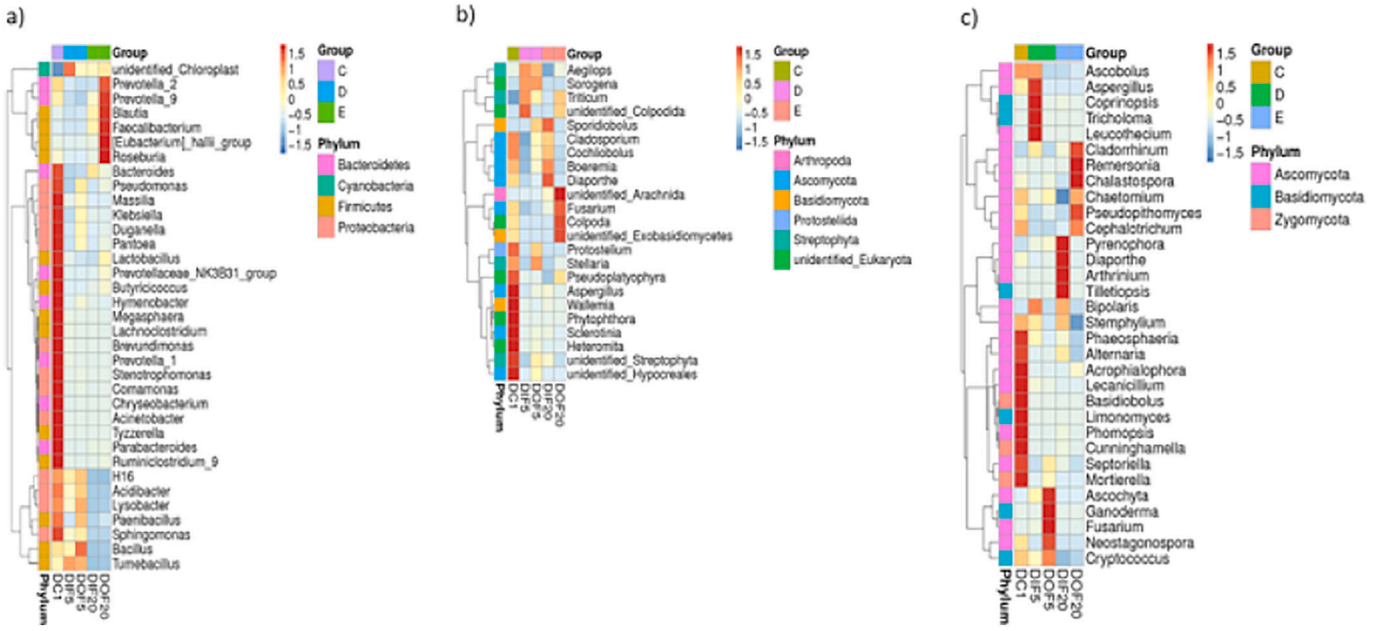


Fig. 3. Taxonomic abundance cluster heatmap for a) bacterial 16S, b) fungal 18S and c) ITS conservative regions plotted by sample name on the X-axis and the Y-axis represents the genus. The absolute value of 'z' represents the distance between the raw score and the mean of the standard deviation. 'Z' is negative when the raw score is below the mean, and vice versa. DC1 - untreated control grains, DIF5/DOF5 and DIF20/DOF20 - directly/indirectly plasma treated grains for 5 and 20 min, respectively.

observed species and population diversity decreased with increasing treatment time from 5 to 20 min (Fig. 4a and c, respectively). However, with 18S sequencing, treatment time had no impact on average number of OTUs detected, and species diversity was slightly higher after 20 min than after 5 min of treatment. Other indices, such as Simpson, Chao1 and ACE, as well as phylogenic diversity whole tree index, decreased with plasma treatment (Supplementary Table 2). The Venn and Flower diagram (Supplementary Fig. 2) illustrate that the number of unique bacterial and fungal species on the treated samples was always lower than that of the control. In total, 92 bacterial and 45/97 (18S/ITS, respectively) fungal OTUs were observed in common between all samples.

3.1.4. Beta diversity indices

Beta diversity analysis evaluates differences in species complexity of samples. Weighted Unifrac and Unweighted Unifrac distances were selected to measure the dissimilarity coefficient between pairwise samples. The heatmap based on the Weighted Unifrac and Unweighted Unifrac distances is presented in Fig. 5. Using Unweighted Unifrac distance, bacterial OTUs of the control sample differed from those of the treated samples and the diversity of 5 min-treated samples differed from 20 min treated samples (Fig. 5a). Fungal population was more diverse on the control within each sample pair, with OTUs observed with ITS sequencing (Fig. 5c). However, no apparent difference between control and indirectly treated samples was observed with 18S for 5 min (Fig. 5b). PCA based on bacterial and fungal OTUs clearly divided control and treated samples (Supplementary Fig. 3).

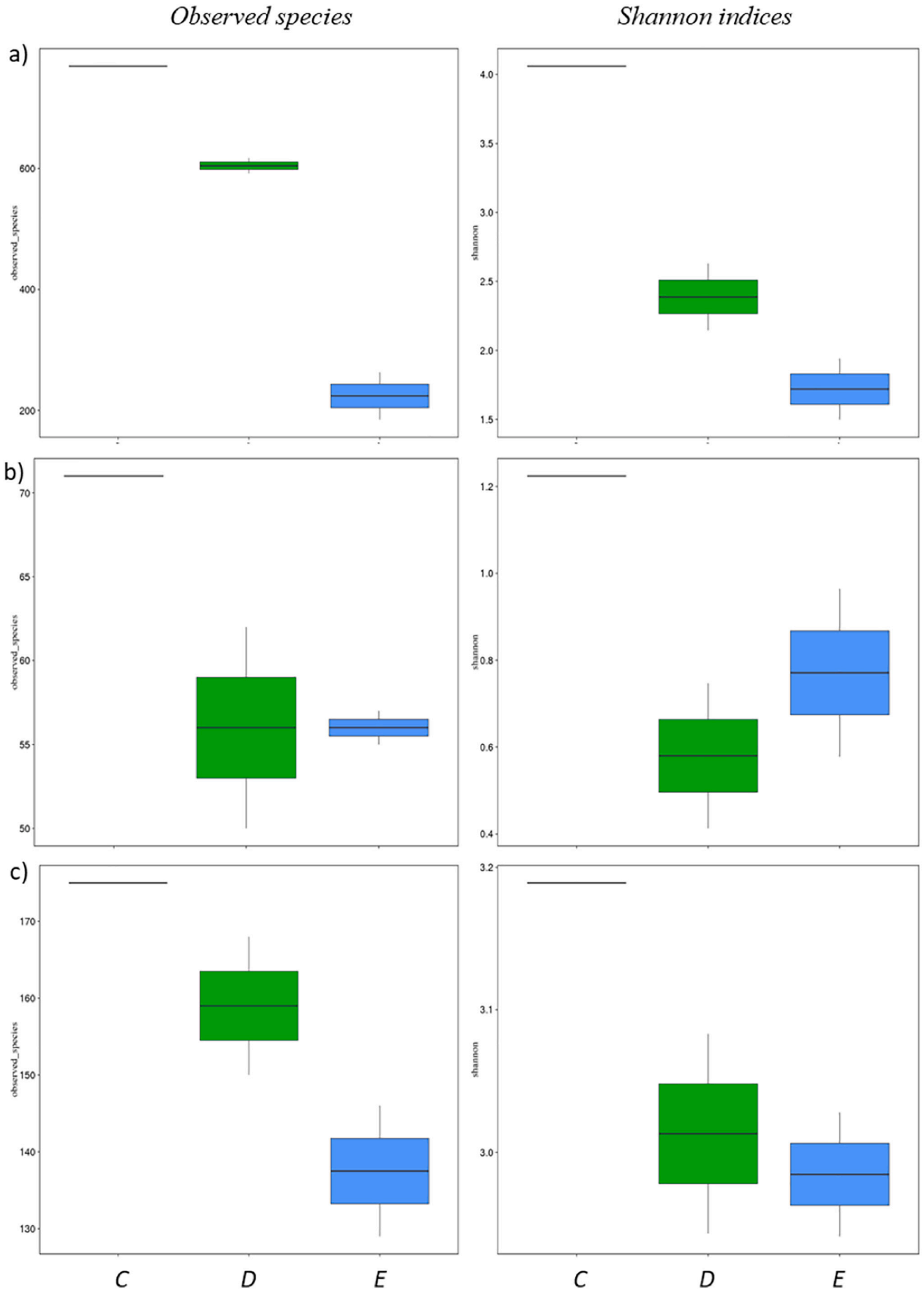
3.2. Effect of ACP on inoculated grain

3.2.1. Susceptibility profile

The efficacy of ACP treatment against a range of challenge microorganisms inoculated on wheat grains was investigated. The surviving microbial populations and reductions of these challenge microorganisms are presented in Table 1a and b, respectively. ACP treatment significantly reduced the level of all microorganisms attached on the surface of wheat grains ($P < 0.05$), however, the type of microorganism in conjunction with treatment variable parameters, influenced the inactivation effects. For instance, the mode of exposure did not significantly influence the efficacy of treatment in the case of *P. verrucosum* and *P. citrinum* when longer treatment (20 min) was applied. However, higher inactivation levels for *A. niger*, *P. chrysogenum*, *A. flavus* and endospores and vegetative cells of *B. atrophaeus* were obtained after 20 min of direct mode of treatment as compared to indirect mode. Extending the direct treatment time from 5 to 20 min did not influence plasma inactivation effects against *A. candidus*, whereas longer direct plasma treatment duration significantly affected all other microorganisms tested. Overall, reduction levels for *B. atrophaeus* vegetative cells were higher than for all fungal species tested, whereas *B. atrophaeus* spores were the most resistant to ACP among all microorganisms tested.

3.2.2. Capacity of ACP to induce bacterial sporulation

To determine the effect of direct/indirect ACP treatment on sporulation ability of *B. atrophaeus*, the concentration of vegetative cells and spores on wheat grains were monitored on days 0 (after treatment and 24 h PTRT), 7 and 21 of grain incubation at two different temperatures, i.e. 15 °C and 30 °C (Fig. 6a and b, and Supplementary Table 3a and b, respectively). The percentage values of spores in total bacterial counts (CFU/g) during incubation were also calculated and are shown in the right upper corner of each corresponding graph. No spores were recovered from grains immediately after plasma treatment. The number of spores on the control grains increased from 0 to 0.91 ± 0.74 $\log_{10}$ CFU/g on day 0, reaching a maximum of 3.24 ± 0.04 $\log_{10}$ CFU/g by day 21, which did not exceed 0.13% of total bacterial counts, regardless of the temperature condition studied. The ACP treatment influenced cell sporulation. On day 0, after 5 and 20 min of indirect treatment and PTRT, the population of spores increased to 0.41 ± 0.08 and 0.48 ± 0.17 $\log_{10}$ CFU/g, respectively, and no spores were recovered from the directly treated grains for any duration of treatment. However, on day 0, the calculated percentage spores after 20 min of indirect treatment was significantly higher than percentage spores on the control ($P < 0.05$). The capacity of cells to sporulate was influenced by the incubation temperatures used. On day 7, samples exposed to plasma and incubated at 15 °C showed significantly higher percentage spores ($P < 0.05$) as compared to untreated control grains (except for indirect



(caption on next page)

Fig. 4. Boxplots of Alpha Diversity indices (observed species and Shannon indices) for a) 16S, b) 18S and c) ITS conservative regions between groups, such as C: Control sample, D: 5-min treated (direct and indirect modes) and E: 20-min treated (direct and indirect modes) group of samples.

5-min treatment), and only 20 min of direct treatment resulted in further increase in percentage spores on day 21. On day 7, percentage spores on all treated samples incubated at 30 °C was significantly higher than percentage value of the control and higher than percentage spores on samples incubated at 15 °C; however, a clear trend towards reduction of percentage spores was evident on day 21, nullifying the temperature effect on sporulation process when longer incubation time was applied.

3.2.3. Microbial resistance studies

Resistance studies were performed to assess if sub-lethal ACP treatment (5 min) can have a selective effect and therefore induce protection mechanisms in *B. atrophaeus* and *A. flavus* to subsequent treatment. For both *B. atrophaeus* and *A. flavus* there was no significant difference between the reduction factors obtained for each of the five consecutive cycles of either direct or indirect plasma treatments. An average reduction of 2.57 ± 0.46 $\log_{10}$ and 2.47 ± 0.28 $\log_{10}$ units were obtained for cells of *B. atrophaeus* after direct and indirect exposure, respectively (Fig. 7a). For spores of *A. flavus*, the average reduction factors were 1.60 ± 0.41 and 1.53 ± 0.45 $\log_{10}$ units after direct and indirect modes of plasma exposure, respectively (Fig. 7b).

4. Discussion

The importance of cereal grains and their products makes their decontamination and infection control a persistent concern. Current technologies applied for decontamination of cereals can negatively affect their quality and technological properties, as well as generate harmful environmental impacts (A. Los et al., 2018b; Yadav et al., 2014). Cold plasma technology is under wide investigation for controlling seed-borne microbial contamination (Randeniya and De Groot, 2015; Scholtz et al., 2019). There are complex polymicrobial communities on seeds surfaces and interiors. Therefore, a detailed knowledge of what the characteristic microbial community of grains and/or cereal produce is required to understand the holistic effects of applying a novel biodecontamination treatment. The focus of this work was to study the influence of cold plasma on wheat grains microbial community structure and composition and to evaluate the efficiency of treatment against challenge microorganisms associated with grains quality defects. Additionally, the microbial sporulation and resistance profile to ACP using real food matrices were assessed. To define and compare microbial communities present in organic wheat grains before and after plasma treatment, amplicon-based HTS was used for comprehensive assessment of interactions between ACP and different microbial community components occurring immediately after treatment. The HTS analysis identified diverse bacterial and fungal communities present in grain microbiome. The most abundant bacterial phyla were *Proteobacteria*, followed by *Cyanobacteria,* with a lower frequency of *Firmicutes* and *Bacteroidetes*. The dominant classified genera were assigned to *Pantoea*, *Pseudomonas*, *Massilia*, and members belonging to class *Bacilli (Bacillus, Paenibacillus, Tumebacillus, Lactobacillus)*. *Ascomycota* was the dominant fungal phylum determined using both 18S and ITS approach, with the highest number of reads aligned to non-mycotoxigenic *Cochliobolus*, *Boeremia*, *Cladosporium* and mycotoxigenic *Aspergillus*, *Fusarium*, *Alternaria.* These genera are phytopathogens, known to infect plants including wheat, cause spoilage in stored grains, produce mycotoxins and pose human and animal health at serious risk (Marin-Felix et al., 2017; Neupane et al., 2010; Yuan et al., 2018). These findings are in agreement with other reports (Hertz et al., 2016; Solanki et al., 2019), which showed similar wheat microbiome identified by culture based and/or HTS techniques. The relative taxonomic abundance results revealed that a shift occurred in the microbial communities on the treated samples. Although meta-genomic approach was unable to distinguish between viable and dead microbial populations within microbiome (Cao et al., 2017), the results demonstrated that some bacterial species, for example *Acinetobacter*, *Lysobacter* of *Gammaproteobacteria*, were less abundant or not detected after plasma treatment (snapshot of Krona display presented in Supplementary Fig. 4). In addition, the number of unique bacterial species was always higher on the control than on plasma treated samples and the bacterial diversity of the treated samples decreased with increasing treatment time. However, result from 18S/ITS OTUs demonstrated that although relative abundance of fungi also decreased with plasma treatment, the fungal communities appeared to be less influenced by the duration of the plasma treatment. This poses considerations for application, where microbiome shifts may need to be considered in terms of integrated pest management. Solanki et al. (2019) demonstrated that phosphine fumigation resulted in changes in the composition of wheat microbiome, showing drastic reduction in bacterial populations, with no effect on either diversity or abundance of fungi. Using culture-based enumeration techniques, our previous studies found that fungi were more sensitive than bacteria to high voltage plasma discharge (80 kV), with fungal populations on wheat grains reduced by 2.5 $\log_{10}$ while aerobic mesophilic bacteria declined by 1.5 $\log_{10}$ CFU/g using direct treatment for 20 min followed by a 24 h post-treatment retention time (A. Los et al., 2018a). Antimicrobial efficacy of direct ACP treatment results

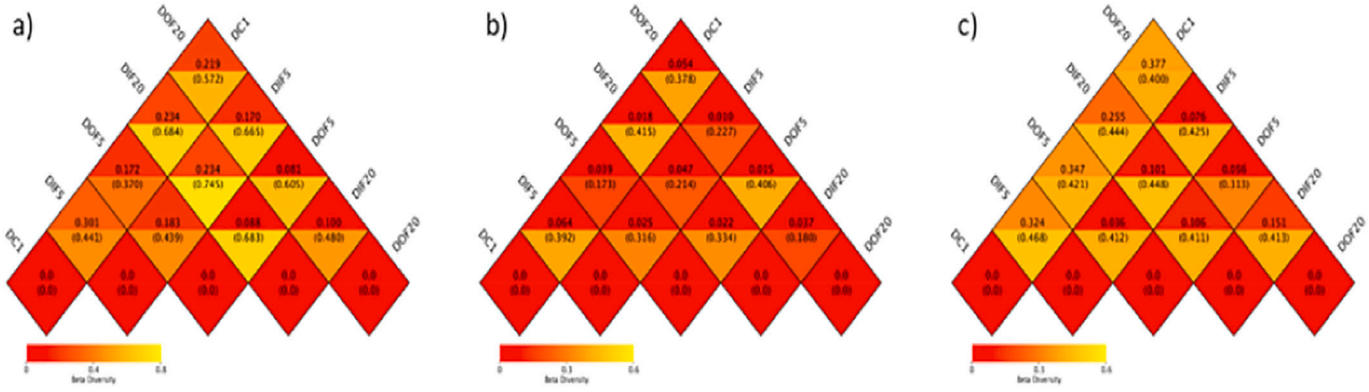


Fig. 5. Beta diversity heatmap based on a) 16S, b) 18S and c) ITS OTUs conservative regions. Each grid represents pairwise dissimilarity coefficient between pairwise samples, in which Weighted Unifrac distance displayed above and Unweighted Unifrac distance conversely. DC1 - untreated control grains, DIF5/DOF5 and DIF20/DOF20 - directly/indirectly plasma treated grains for 5 and 20 min, respectively.

Table 1
Effect of ACP on viability of microorganisms inoculated on wheat grains: (a) total microbial counts and (b) reduction factor.

(a)		Microbial populations, $\log_{10}$ CFU/g							
		A. candidus	*A. niger*	*A. flavus*	*P. chrysogenum*	*P. verrucosum*	*P. citrinum*	*B. atrophaeus*	
								vegetative cells	spores
Control		6.76 ± 0.18^{a}	6.09 ± 0.54^{a}	6.32 ± 0.11^{a}	6.74 ± 0.19^{a}	5.92 ± 0.60^{a}	6.29 ± 0.09^{a}	5.63 ± 0.34^{a}	6.48 ± 0.05^{a}
5 min	Indirect	5.55 ± 0.72^{b}	5.41 ± 0.62^{b}	5.80 ± 0.19^{b}	4.97 ± 0.39^{b}	5.08 ± 0.85^{b}	4.89 ± 0.31^{b}	3.38 ± 0.17^{b}	6.07 ± 0.03^{b}
	Direct	4.62 ± 0.64^{c}	5.38 ± 0.65^{b,c}	5.73 ± 0.26^{b,c}	4.85 ± 0.44^{b}	5.15 ± 0.52^{b}	4.68 ± 0.29^{b}	3.25 ± 0.33^{b}	5.76 ± 0.17^{c}
20 min	Indirect	4.44 ± 0.33^{c}	5.14 ± 0.02^{b,c}	5.01 ± 0.20^{c}	4.80 ± 0.23^{b}	4.19 ± 0.34^{c}	3.95 ± 0.27^{c}	2.55 ± 0.44^{c}	5.89 ± 0.11^{c}
	Direct	3.99 ± 0.59^{c}	4.53 ± 0.69^{d}	4.61 ± 0.39^{d}	4.37 ± 0.25^{c}	4.18 ± 0.45^{c}	3.72 ± 0.15^{c}	1.75 ± 0.69^{d}	5.55 ± 0.14^{d}

(b)		Reduction factor ($\log_{10}$)			
		5 min		20 min	
Microorganism		Indirect	Direct	Indirect	Direct
A. candidus		1.20 ± 0.66^{c}	2.14 ± 0.59^{d}	2.32 ± 0.30^{d}	2.77 ± 0.54^{e}
A. niger		0.69 ± 0.56^{a,b}	0.71 ± 0.59^{a}	0.96 ± 0.02^{b}	1.56 ± 0.63^{b}
A. flavus		1.35 ± 0.28^{b,c}	1.47 ± 0.40^{a,b}	1.52 ± 0.21^{c}	1.95 ± 0.23^{c,d}
P. chrysogenum		0.95 ± 0.35^{b,c}	1.01 ± 0.24^{a,b}	1.74 ± 0.18^{c}	2.13 ± 0.35^{c,d}
P. verrucosum		0.89 ± 0.71^{c}	0.77 ± 0.47^{b,c}	1.73 ± 0.31^{c}	1.74 ± 0.41^{b,c}
P. citrinum		1.40 ± 0.29^{c}	1.61 ± 0.26^{c}	2.34 ± 0.25^{d}	2.57 ± 0.14^{d,e}
B. atrophaeus	Vegetative cells	2.25 ± 0.15^{d}	2.38 ± 0.30^{d}	3.08 ± 0.41^{e}	3.88 ± 0.63^{f}
	Spores	0.41 ± 0.03^{a}	0.73 ± 0.16^{a}	0.60 ± 0.10^{a}	0.94 ± 0.12^{a}

Different letters within each column indicate a significant difference at the 0.05 level for each microorganism tested. Experiments were performed in duplicate and replicated three times ($n = 6$).

from a combination of reactive species formed during plasma generation, as well as additional physical processes generated during plasma discharges, such as a high electric field, overpressure shock waves, and intense UV radiation, which are known to inactivate microorganisms by DNA or RNA damage. During indirect ACP treatment, antimicrobial efficacy is dependent primarily on the reactive species (Schnabel and Schmidt, 2017). However, many species within microbial communities are not culturable, as demonstrated by plate recovery method in the current work. Therefore, classical culture based approaches may not reveal the complete changes in the microbial diversity and if not accompanied by a genomic approach, may not reflect the real impact of plasma processing on the microbiome and consequent quality and safety profile of wheat. In this study, many of the microbial species recovered by culture method were not identified by MALDTOF MS, which could possibly be due to limitations in the reference mass spectra of the MS database. Interestingly, culturing enabled detection of *Penicillium* species, which were not described by HTS. This could be due to many factors including sample preparation, incomplete cell lysis and therefore limited DNA extraction efficiency (Mayo et al., 2014) and variation in the microbial genome between the grain samples. However, a good correlation between culture/molecular-based and HTS analyses was demonstrated by Solanki et al. (2019).

Current HTS data indicated that air-based plasma treatment can be tailored for grains preservation; however, interactions between different members of the microbiome should not be ignored. Cereal grains harbor diverse microbial communities, which can play different roles in grain quality by either infecting or protecting the crop (Links et al., 2014; Wachowska et al., 2017). Links et al. (2014) demonstrated that *Pantoea agglomerans* isolated from wheat seeds had antagonistic effects on *Alternaria* spp. Our preliminary results demonstrated that several wheat grain isolates of class *Bacilli* suppressed the growth of fungi of the genus *Fusarium* (Supplementary Fig. 5), pointing to a means for ACP assisted bio-control. *Bacillus* spp. are also known for their growth promotion effects on wheat plant (Akinrinlola et al., 2018). Importantly, our current results indicated that the relative abundance of *Bacilli* increased from 45% (of *Firmicutes*) on the control to a dominant position of 80/93% after direct/indirect 5-min treatment, respectively. However, a reduction in the population to 5/7% after longer, 20 min, of plasma exposure occurred, demonstrating the importance of understanding the relationship between critical process parameters and microflora responses. Furthermore, there was no substantial change in the relative abundance levels of *Pantoea* observed due to extended plasma exposure. The levels of *Pseudomonas* spp., which are known for their ability to inhibit fungal plant pathogens (Wachowska et al., 2017), increased from the control 84% of *Pseudomonadales*, to 100% dominance for plasma treated samples, while the remaining taxa belonging to *Acinetobacter* (14% of *Pseudomonadales*) disappeared post treatment. Some species of *Pseudomonas* and *Acinetobacter* are both human and phyto pathogens (Lee et al., 2017; Solanki et al., 2019). Therefore, to adopt this technology for microbiological control of stored agricultural products, the characterization of long-term effects of ACP on the composition of the grain microbiome is required, to ensure minimal non-target biological effects in response to sustainable integrated pest management requirements.

ACP treatment efficacy for control of potentially pathogenic fungi artificially inoculated on wheat grains used both reference culture collection strains and strains isolated from commercially available organic wheat grains (*Aspergillus* and *Penicillium* spp.). *B. atrophaeus* spores, identified in our previous work as the most resistant microbial form to ACP treatment (Los et al., 2018), was used as an indicator strain for assessment of antimicrobial activity of treatment. Both *Aspergillus* and *Penicillium* are major mycotoxin-producing fungi in grains, which are usually classified as “storage” fungi as they invade grains stored in unsuitable temperature and moisture conditions (Bullerman and Bianchini, 2009). ACP treatment significantly reduced microbial population on wheat grains with the highest efficiency recorded against *B. atrophaeus* vegetative cells and the lowest against *B. atrophaeus* spores, with maximal reduction factors of 3.88 and 0.94 $\log_{10}$ CFU/g, respectively, achieved using 20 min treatment time. Maximal reduction levels for all tested *Aspergillus* and *Penicillium* spp. were in range of 1.56–2.77 $\log_{10}$. As investigated in our other study, ACP treatment of *A. flavus* spores and biofilms results in significant decreases of the remaining cell counts and viability, as well as the release of intracellular material such as MDA (product of membrane lipid peroxidation), DNA and proteins,

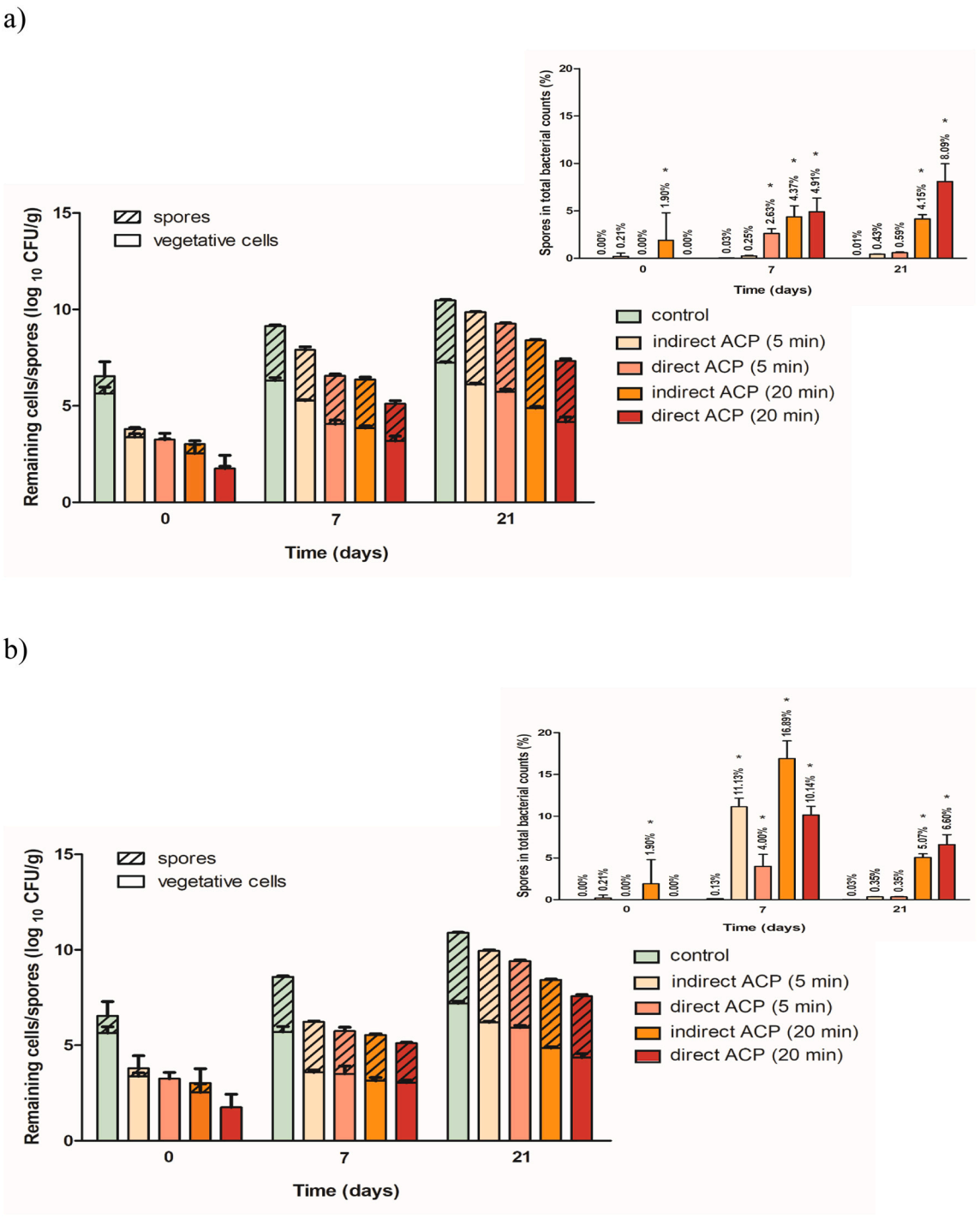


Fig. 6. Effect of ACP direct/indirect treatment on *B. atrophaeus* spore and vegetative cells counts on wheat grains during extended incubation time at a) 15 °C and b) 30 °C. Error bars show the standard deviation. Asterisks indicate a significant difference between the control and ACP-treated samples ($p < 0.05$). All experiments were performed in duplicate and repeated at least three times ($n = 6$).

suggesting partial or total disruption of cell walls leading to compromised cell wall integrity and, subsequently, cell leakage. Pronounced effects of plasma on both external surface elements and internal structures of fungal cells were also observed (Los et al., 2020).

The different susceptibility of *Bacillus* spores towards ACP treatment as compared to fungal spores was demonstrated in other reports. Park et al. (2003) showed the sporicidal effect of atmospheric pressure microwave-induced argon plasma, where an initial count of 10^7 CFU/ml *B. subtilis* spores were sterilized in 20 s, whereas spores of *P. citrinum* were deactivated within 1 s of the plasma exposure. Using a nitrogen gas plasma, Kim et al. (2014) reduced *A. flavus* counts in red pepper powder by 2.5 ± 0.3 $\log_{10}$ CFU/g in 20 min, but did not obtain a reduction of *B. cereus* spores. The lower resistance of fungal spores vs. bacterial spores can be explained by numerous differences between them, including fundamental distinctions between cell structure, type and the role they play in the life cycle of each type of microorganism. Unlike bacterial spores, fungal spores are part of the normal life cycle of fungi, therefore, they are less resistant to chemicals and adverse environmental conditions (Clontz, 2008). Often considered as the beginning and the end of the development cycle of fungi, fungal spores are

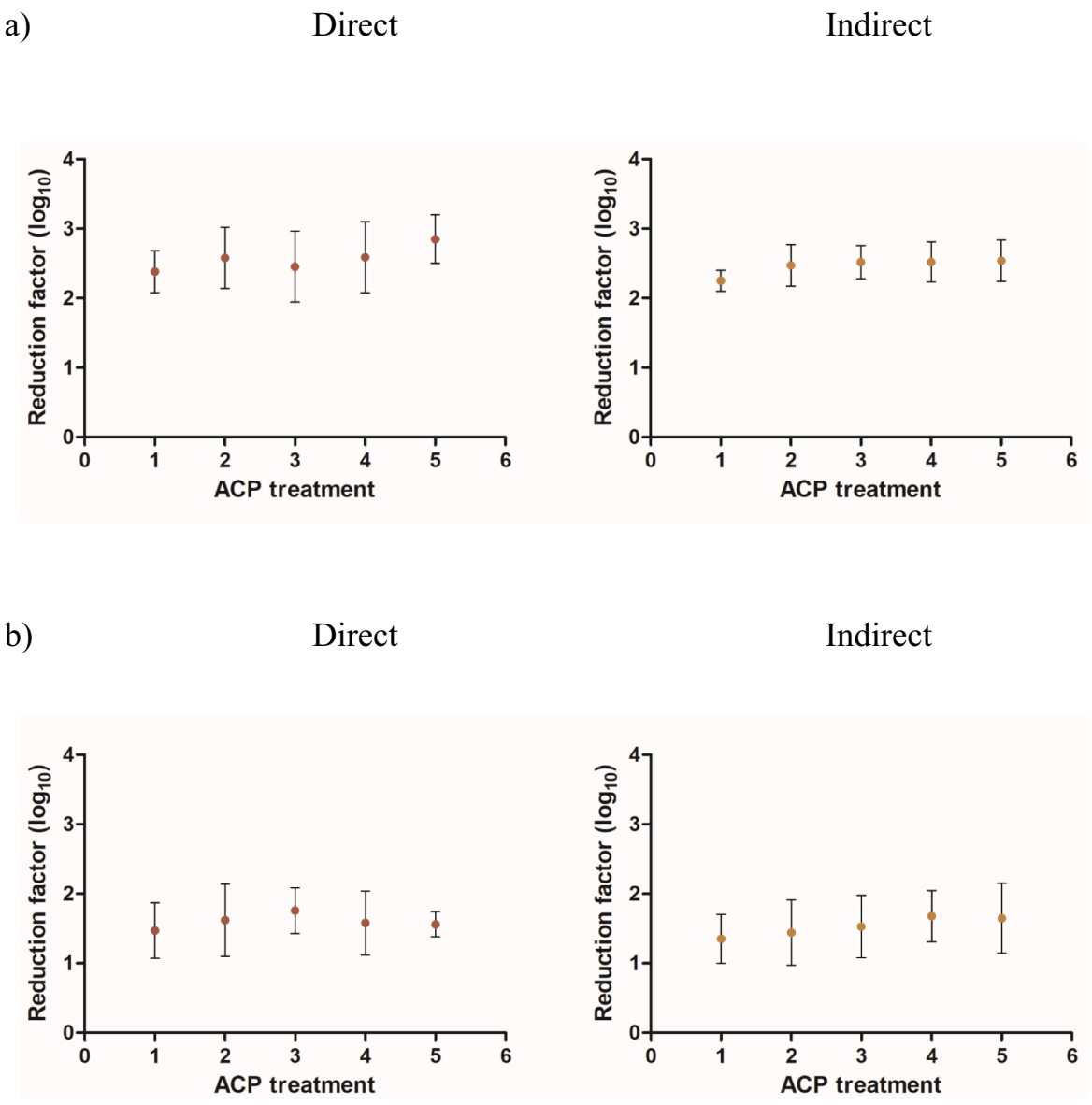


Fig. 7. Reduction factor of inoculated a) *B. atrophaeus* and b) *A. flavus* after repeated exposure of on wheat grains to 5 min of direct/indirect ACP treatment. Error bars represent standard deviation. All experiments were performed in duplicate and repeated at least three times (n = 6).

characterised by a dormant state, in which their metabolic activities decrease to a large extent, however, they still retain some respiratory activity and use functional links of the metabolic chain (El-Enshasy, 2007). Bacterial sporulation, on the other hand, is an important survival strategy activated under adverse environmental conditions. The process of differentiation of vegetative cells into spores can be triggered by multiple environmental signals, such as nutrient deprivation, high mineral composition, neutral pH, temperature, and high cell density (Sella et al., 2014). During sporulation of bacteria, vegetative cells undergo series of profound biochemical changes. This process is not a part of the reproductive cycle, but aims to create a cell type, which is highly resistant to adverse environments, including lack of moisture or essential nutrients, toxic chemicals, radiation and high temperatures. Given the inert metabolic state in addition to protective multi-layered structure and unique composition, this cell type can survive for extended periods with little or no nutrients, and yet remains poised to return to life if nutrients become available (Eissa et al., 2014; Setlow, 2006). Total elimination of bacterial vegetative cells is possible with mild disinfection or sterilisation methods, whereas the same treatment may be completely ineffective against bacterial spores. Once formed, the complete inactivation of spores without compromising the sensory qualities of food proves to be an ongoing challenge (Sinnela et al., 2019).

As bacterial sporulation is an important survival strategy under growth restrictive environmental conditions, it was important to determine the influence of plasma treatment on the formation of spores. The influence on the ability of *B. atrophaeus* to sporulate was quantitatively assessed by exposing vegetative cells inoculated on wheat grains to sub-lethal plasma dose (5 min) and further subjecting treated samples to an extended incubation at 15 °C and 30 °C. Regardless of the temperature, the number of CFUs for both vegetative cells and spores for all samples gradually increased at a significant rate, reaching the maximum on day 21. However, a higher percentage of spores was recovered from ACP treated samples than from untreated samples. The precise mechanism underlying plasma-mediated initiation of sporulation, as well as resultant spore properties and their resistance could provide a target for intervention, to ensure safe application of this technology for agri-food processing where bacterial spore formation is a risk.

The ability of both prokaryotic and eukaryotic organisms to survive

in extreme, rapidly changing and potentially damaging conditions depends on development of sophisticated stress response mechanisms (Giuliodori et al., 2007; Smith et al., 2010). The adaptive capacity of microorganisms favouring selection of naturally occurring resistant variants and horizontal gene transfer processes are worldwide concerns. Antibiotics, especially when present at sublethal concentrations, are responsible for increasing genetic variation and therefore participating in the emergence of antibiotic resistant strains (Blázquez et al., 2012). The generation of reactive species in the plasma discharge has been shown to illicit stress responses in prokaryotes and eukaryotes in terms of the generation of intracellular reactive oxygen species (Han et al., 2016) and the transcription of stress responsive genes, such as those involved in SOS response or DNA repair (Sharma et al., 2009). In the present study, a 5-fold repeated ACP treatment did not induce resistance in either bacterial vegetative cells of *B. atrophaeus* or fungal spores of *A. flavus* inoculated on wheat grains. These results are therefore in agreement with results reported by Zimmermann (2012), where no natural or acquired resistance in *Escherichia coli* (*E. coli*) and *Enterococcus mundtii* in response to ACP treatment occurred. Similarly, Matthes et al. (2014) observed that a 6-fold repeated plasma treatment did not induce resistance in *Staphylococcus aureus* (*S. aureus*) embedded in biofilms. As reported by Brun et al. (2015), repeated exposure to helium plasma for 30 s once daily for 10 days did not induce resistance to plasma in populations of *S. aureus* and *E. coli*. This work demonstrated that cold plasma could be a promising tool for addressing both bacterial and fungal bio-contaminants of cereal grains to enhance grains preservation. As a technology harnessing diverse and multiple mechanisms of action, ACP could address bio-contamination and sustainability issues across the diverse uses of grains within the agri-food chains.

5. Conclusion

In conclusion, high voltage contained ACP treatment had a significant impact on the structure and composition of wheat microbiome. Higher microbial diversity and abundance was found on the untreated control as compared to plasma treated grain samples. ACP treatment was effective against a range of challenge pathogens artificially inoculated on the surface of wheat grains, which demonstrates the potential of cold plasma for ensuring stored grains biodecontamination. Repeated ACP treatment did not induce resistance in either bacterial vegetative cells of *B. atrophaeus* or fungal spores of *A. flavus* inoculated on wheat grains. Further studies are necessary to understand the mechanisms of sporulation mediated by cold plasma. Because of the impacts noted on the grains microbiome, further studies are recommended to investigate the change in microbial community profile during long term contained grain storage scenarios to understand the potential of ACP to promote advantageous bio-control as a means of sustainable integrated pest management.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2020.108889.

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